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Diffuse Large B Cell Lymphoma Shows Distinct Methylation Profiles of the Tumor Suppressor Genes among the Non-Hodgkin's Lymphomas

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Background : Aberrant methylation of CpG islands in promoter regions is one of the major mechanisms for silencing of tumor suppressor genes in various types of human cancers including non-Hodgkin's lymphomas (NHL). In this study, we investigated the aberrant promoter methylation status of known or suspected tumor suppressor genes in NHLs and compared the methylation profiles between B-cell and T/NK-cell NHLs. **Methods :** 54 cases of B-cell NHLs and 16 cases of T/NK-cell NHLs were examined for the methylation status of eight genes using methylation specific PCR. **Results :** CpG islands methylation was variously found in eight genes as follows; *DAPK* (71%), *MT1G* (70%), *p16* (53%), *CDH1* (53%), *THBS1* (56%), *MGMT* (27.1%), *COX2* (13%), and *RUNX3* (11.4%). In six cases (8 %), methylation was not observed in any of these genes. Overall methylation index of B-cell NHLs (0.48) was significantly higher than that of T/NK-cell NHLs (0.32). Of eight genes tested, *THBS1* and *CDH1* methylations were much more prominent in diffuse large B-cell lymphomas than in T/NK-cell NHLs or other B-cell NHLs. **Conclusion :** This study suggests that aberrant CpG island methylation is a frequent event in NHLs, and diffuse large B-cell lymphomas show overlapping but distinct methylation profiles.

Key Words : Lymphoma, non-Hodgkin; DNA methylation; Genes, tumor suppressor

Hypermethylation of CpG islands in the promoter regions is an important mechanism of gene silencing for tumor suppressor genes (TSG).[1] The aberrant methylation of the CpG islands has been correlated with loss of gene expression, and DNA methylation provides an alternative pathway for gene deletion or mutation for the loss of TSG function.[1,2] Aberrant promoter methylation has been described in several kinds of malignant tumors, and each type of tumor may have its own distinct pattern of methylation.[2-4] Hematological neoplasms are also known to have very different hypermethylation profiles than those of other solid tumors.[4] To date, there haven't been extensive studies about aberrant promoter methylation of TSGs in non-Hodgkin's lymphomas (NHL); only a limited numbers of TSGs have been tested and their analysis has been restricted to certain types of NHLs.[5-9]

In this study, we explored the prevalence of aberrant methylation in a selected panel of eight TSGs that are known or rarely known to exist in lymphomas using methylation specific PCR. The selected eight TSGs are known to be involved in cell cycle regulation (*p16*, *COX2*),[10,11] DNA repair (*MGMT*),[7] apoptosis (*DAPK*, *RUNX3*),[8,12] angiogenesis inhibitor (*THBS1*),[13] invasion and metastasis (*CDH1*)[11] and cell proliferation (*MT1G*).[14] The methylation status was examined in all the enrolled lymphoma cases and this was analyzed specifically according to the cellular origins (B-cells or T/NK-cells) of the NHLs.

MATERIALS AND METHODS

Tumor samples and DNA preparation

Seventy tumor samples that were diagnosed as NHLs were obtained from the archives of Seoul National University Hospital and Seoul National University Boramae Hospital, Seoul, Korea between 1999 and 2003. The tumor samples were formalin-fixed, paraffin-embedded tissues derived from lymph nodes, gastrointestinal tracts, nasal cavity, tonsils, brain or other involved organs. There were 54 cases of B-cell NHLs and 16 cases of T/NK-cell NHLs. The subclassification of lymphomas,

according to the WHO classification,[15] is summarized in Table 1. The control samples included the DNAs obtained from peripheral blood lymphocytes from two healthy adult volunteers and three lymph node tissues diagnosed as reactive hyperplasia. The genomic DNA was isolated using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The institutional review board of Seoul National University Boramae Hospital approved this study.

Bisulphite modifications

DNAs were subjected to sodium bisulphite modification, as described previously.[11] In brief, 40 μL of DNA (2 μg) was denatured at 97℃ for 6 min, it was quickly centrifuged and then chilled on ice. Ten microliters of 1 M NaOH was then added and the mixture was stored at room temperature for 15 min. Five hundred fifty microliters of 3.5 M sodium bisulphite and 1 mM hydroquinone mixture was then added to the denatured DNA, which was then stored at 55℃ for 16 h. The treated DNA was purified with a JETSORB gel extraction kit (Genomed, Bad Oeynhausen, Germany) and desulphonated with 0.3 M NaOH at room temperature for 10 min. After adding three volumes of 100% cold ethanol and a two-thirds volume of 7.5 M ammonium acetate and storing at -20℃ for 12 h, the precipitated DNA was centrifuged. After washing in 70% ethanol and drying, it was dissolved in 10 mM Tris buffer.

Methylation-specific PCR (MSP)

A panel of eight genes was analyzed for the methylation status using MSP. The primer sequences of each gene, the product size, the annealing temperature, and references are given in Table 2. All the PCR amplifications were performed using bisulphate-modified DNA (30-50 ng), primers (10 pmol each), dNTPs (1 mM each), 10X standard PCR buffer (Qiagen) and 0.5 U of HotStarTaq Plus DNA polymerase (Qiagen) in a volume of 20 μL. The reactions were hot-started at 95℃ for 5 min, followed by 35 cycles at 94℃ (30 s per cycles), with the annealing temperature being specific for each reaction (30 s per cycle), and 72℃ (30 s per cycle), and a final extension step was done at 72℃ for

Table 1. Case summary of non-Hodgkin's lymphomas (NHL) according to WHO Classification

B-cell NHL (n=54)		T/NK-cell NHL (n=16)	
Burkitt's lymphoma	2	T-lymphoblastic lymphoma	1
Diffuse large B-cell lymphoma	46	Angioimmunoblastic T-cell lymphoma	2
Follicular lymphoma	1	Anaplastic large cell lymphoma	5
Mantle cell lymphoma	2	Extranodal NK/T-cell lymphoma	4
Extranodal marginal zone B-cell lymphoma	2	Peripheral T-cell lymphoma, unspecified	4
Small lymphocytic lymphoma	1		

Table 2. Primer sequences and PCR conditions for methylation-specific PCR analysis

Primer name		Primer sequence (5'-3')		Product size (bp)	Annealing temperature (°C)	Reference
		Forward	Reverse			
CDH1	m	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	97	53	11
	u	TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	91	59	
COX2	m	TTAGATACGGCGGCGGCGGC	TCTTTACCCGAACGCTTCCG	161	61	10
	u	ATAGATTAGATATGGTGGTGG TGGT	CACAATCTTTACCCAAACACTTCCA	171	61	
DAPK	m	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	98	60	8
	u	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	98	60	
MGMT	m	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	81	65	7
	u	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	93	59	
MT1G	m	TGCGAAAGGGGTCGTTTTGC	GCGATCCCGACCTAAACTATACG	93	59	14
	u	GTGAGTTGGTGTGAAAGGGGTT	CCACACCACCCACAATCCCA	113	59	
P16	m	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	65	11
	u	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	60	
RUNX3	m	TTCGTTTATTTTGTCGTCGT	CGCTATTATACGTATTCCCG	100	55	12
	u	TTTGGGTTTATGGGAATATG	TTCTCACAACAACAACAACC	120	55	
THBS1	m	TGCGAGCGTTTTTTTAAATGC	TAAACTCGCAAACCAACTCG	74	62	13
	u	GTTTGGTTGTTGTTTATTGGTTG	CCTAAACTCACAAACCAACTCA	115	62	

m, methylated sequence; u, unmethylated sequence.

10 min in the PTC200 thermal cycler (MJ research, Waltham, MA, USA). The PCR products (5 μL) were electrophoresed on 2% agarose gels and they were visualized after staining with ethidium bromide. For each MSP reaction, we used normal lymphocyte DNA treated with Sss1 methyltransferase (New England Biolabs, Beverly, MA, USA) and distilled water without template DNA as a positive and negative control, respectively.

Statistical analysis

The frequencies of methylation for the two groups were compared using Fisher's exact test or the χ^2 test. The methylation index (MI), as a reflection of the methylation status of all of the tested genes, is defined as the total number of genes methylated divided by the total number of genes analyzed. We calculated the MIs for each case to compare the extent of methylation for the panel of the examined genes[16] and then we determined the mean for the different groups. Statistical analysis of MI between two variables was performed using the Mann-Whitney U nonparametric test. For all tests, p value <0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS software (SPSS for windows Release, version 12.0, SpSS, Chicago, IL, USA).

RESULTS

None of the eight genes had methylation detected in the five control samples. However, methylation for these genes was common in NHLs (examples in Fig. 1). Out of the 70 cases of NHLs,

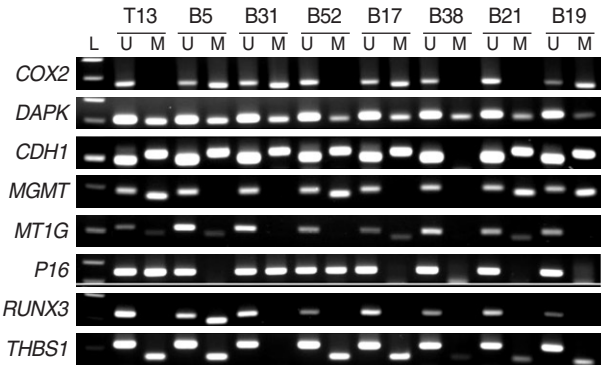

Fig. 1. Methylation specific PCR results for eight genes in non-Hodgkin's lymphomas. The PCR products in lane U indicate the presence of unmethylated alleles, and the products in lane M indicate the presence of methylated alleles.
L, size marker (100 bp DNA ladder).

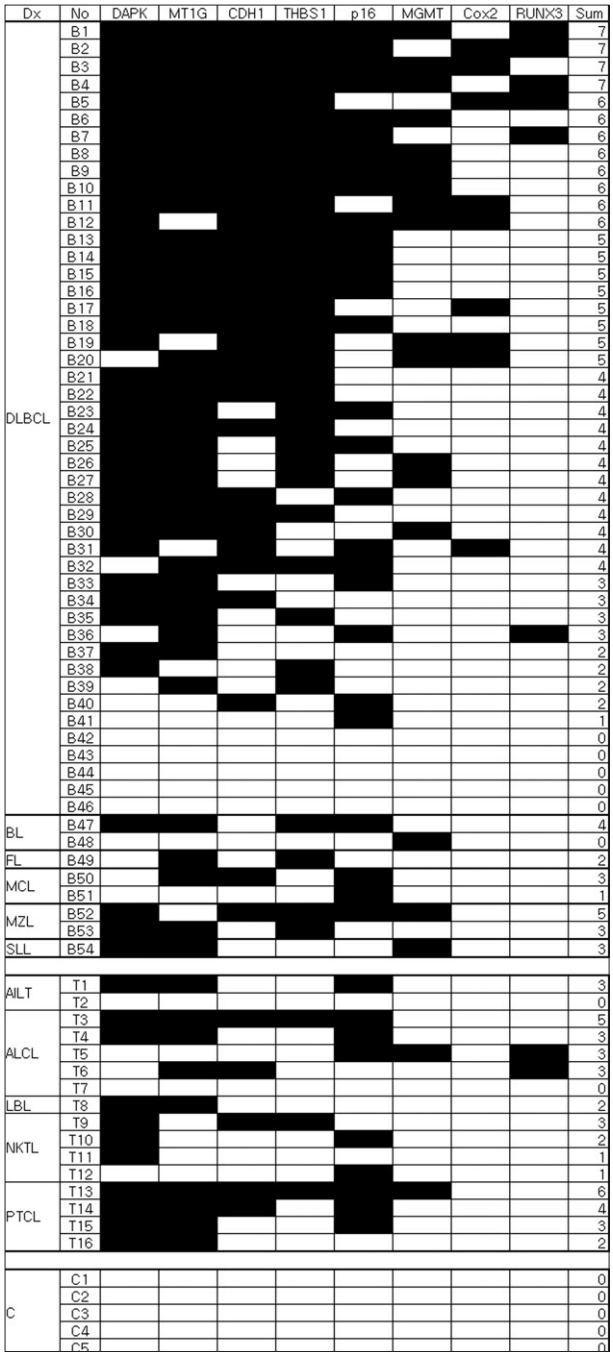

Fig. 2. Summary of the methylation analysis of *DAPK*, *MT1G*, *p16*, *CDH1*, *THBS1*, *MGMT*, *COX2*, and *RUNX3* in non-Hodgkin's lymphoma samples. The filled boxes indicate the presence of methylation and the open boxes indicate the absence of methylation. Dx, diagnosis; No, case number; Sum, the number of methylated genes; DLBCL, diffuse large B-cell lymphoma; MZL, extranodal marginal zone B-cell lymphoma; BL, Burkitt's lymphoma; MCL, mantle cell lymphoma; FL, follicular lymphoma; SLL, small lymphocytic lymphoma; AILT, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large cell lymphoma; NKTL, Extranodal NK/T-cell lymphoma; PTCL, peripheral T cell lymphoma, unspecified; LBL, T-lymphoblastic lymphoma; C, control sample.

Table 3. Methylation frequency of each gene tested in B-cell and T/NK-cell non-Hodgkin's lymphomas (NHL)

	T/NK-cell NHLs (n=16)	B-cell NHLs (n=54)		Total (n=70)
		DLBCL (n=46)	Other B NHLs (n=8)	
CDH1	5 (31.3%)*	30 (65.2%)*,†	2 (25.0%)†	37 (52.9%)
COX2	0	9 (19.6%)	0	9 (12.9%)
DAPK	11 (68.8%)	35 (76.1%)	4 (50.0%)	50 (71.4%)
MGMT	2 (12.5%)	14 (30.4%)	3 (37.5%)	19 (27.1%)
MT1G	9 (56.3%)	35 (76.1%)	5 (62.5%)	49 (70.0%)
P16	9 (56.3%)	24 (52.2%)	4 (50.0%)	37 (52.9%)
RUNX3	2 (12.5%)	6 (13.0%)	0	8 (11.4%)
THBS1	3 (20.0%)‡	32 (69.6%)‡,§	4 (50.0%)§	39 (56.5%)
Methylation index	0.32‖	0.50‖	0.34	

*p, 0.018; †p, 0.033; ‡p, 0.001; §p, 0.033; ‖p, 0.007.

64 cases (91.4%) exhibited aberrant promoter methylation in at least one gene. The detailed results for the methylation of eight genes in each lineage of lymphoma are given in Fig. 2. Each of the eight genes showed methylation of CpG islands in the promoter region at frequencies of 11-71%, and the methylation frequencies of each examined gene are summarized in Table 3. In particular, five genes (*CDH1*, *DAPK*, *MT1G*, *P16* and *THBS1*) were frequently methylated (>50%) in all the NHLs.

Because most of the B-cell NHLs were diffuse large B cell lymphomas (DLBCL), we analyzed the methylation frequencies of DLBCL compared to those of the T/NK-cell lymphomas or the other B-cell lymphomas. Of the eight genes tested, *CDH1* and *THBS1* were more methylated in DLBCLs than that in the T/NK-cell lymphomas or the other B-cell lymphomas (Table 3). In addition, the mean MI of the DLBCLs (0.5) was significantly higher than that of the T/NK-cell NHLs (0.32; p=0.007). The differences of the mean MI among the B cell lymphomas (0.5 for the DLBCLs and 0.34 for the other B-cell NHLs) were statistically insignificant (p=0.055).

DISCUSSION

In this study, we comprehensively investigated aberrant promoter methylation of multiple tumor suppressor genes in NHLs using MSP. Out of these eight genes included in our study, four genes (*DAPK*, *CDH1*, *p16*, and *MGMT*) have been previously reported to be frequently methylated (>20%) in hematological malignancies,[5-9,17] and our findings fall within the published ranges. To the best of our knowledge, the other four genes (*THBS1*, *MT1G*, *RUNX3*, and *C0X2*) we studied have not previously been investigated in detail in NHLs. *THBS1* is a potent inhibitor of angiogenesis and its inactivation by promoter methylation was detected in glioblastoma[18] as well as many other tumors.[6,12,13] *MT1G* encodes a member of the metallothionein (MT) heavy metal binding proteins that are active in metal homeostasis and they have been linked with cell proliferation, and *MT1G* gene methylation was observed in esophagus, kidney, thyroid and prostate carcinomas.[14,19-21] *RUNX3* is one of the genes with a RUNT domain, and *RUNX3* has been reported to be an important tumor suppressor gene in gastric cancer,[22] whereas *C0X2* is the rate-limiting enzyme for the production of prostanoids (prostaglandins and thromboxanes) from arachidonic acid, which is known to be causally involved in colorectal carcinogenesis and gastric cancers.[10,23] Of these four genes, we discovered that the aberrant methylation of *MT1G* and *THBS1* occurred frequently (70.0% and 56.5%, respectively) in NHLs, which suggested that *MT1G* and *THBS1* may also contribute to the pathogenesis of NHLs. However *C0X2* and *RUNX3* were rarely methylated in NHLs (12.9% and 11.4%, respectively).

Our results showing high methylation frequencies (>50%) in five genes (*DAPK*, *MT1G*, *THBS1*, *p16* and *CDH1*) together with high MI indicated that the simultaneous inactivation of multiple genes occurred in the lymphoma samples. These findings may be of potential significance for creating demethylating therapeutic strategies.[24]

Most of the B-cell lymphomas in this study were DLBCL, and we found that the mean MI of the DLBCLs was hignificantly higher than that of the T/NK-cell lymphomas, but the difference between the mean MI of the DLBCL and that of the other B-cell lymphomas was not statistically significant. However, the number of cases of other B-cell lymphomas (8 cases) in this study was relatively smaller than that of the other subgroups, so further studies encompassing many other B-cell lymphomas are needed.

Among the eight genes we examined, *THBS1* and *CDH1* were more frequently methylated in DLBCLs compared to T/NK-cell NHLs or the other B-cell NHLs. Methylation of *THBS1* in tumor cells will theoretically be expected to produce neovascularization or vascular proliferation, but we did not generally observe more prominent vascular components in the DLBCLs than in the other NHLs. There might be some additional pathways related to angiogenesis rather than *THBS1* methylation or there may be other unknown roles for this gene. Further researches should be done regarding this issue. *CDH1* (E-cadherin), as one of the cadherin molecules, may enhance tumor progression and invasion by multiple mechanisms including reduced cell-to-cell

adhesion. The fact that CDH1 was highly methylated in DLBCLs correlated with the aggressive behavior of DLBCLs.

In this study, 5 cases of diffuse large B cell lymphoma showed no methylation in any of the eight tested genes. Three of the cases were from lymph nodes, one from brain and the other was from ileum. There were no significant differences between those methylation-nil cases and the other methylated cases regarding the morphology, the immunohistochemical markers (such as CD10, bcl2, bcl6, mum1, etc) and the proliferation indices (data not shown).

Due to the limited number of cases other than the diffuse large B cell lymphomas, we could not perform a subtype specific methylation analyses among the eight genes in this study. However, this study provides insight for understanding the molecular pathogenesis of NHLs. DLBCLs showed a different and distinct methylation pattern compared to T/NK-NHLs or the other B-cell NHLs, which may reflect the different mechanisms of lymphomagenesis according to their cellular lineage.

REFERENCES

1. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002; 3: 415-28.
2. Costello JF, Fruhwald MC, Smiraglia DJ, *et al*. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet 2000; 24: 132-8.
3. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene 2002; 21: 5427-40.
4. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res 2001; 61: 3225-9.
5. Baur AS, Shaw P, Burri N, Delacretaz F, Bosman FT, Chaubert P. Frequent methylation silencing of p15(INK4b) (MTS2) and p16 (INK4a) (MTS1) in B-cell and T-cell lymphomas. Blood 1999; 94: 1773-81.
6. Chu LC, Eberhart CG, Grossman SA, Herman JG. Epigenetic silencing of multiple genes in primary CNS lymphoma. Int J Cancer 2006; 119: 2487-91.
7. Esteller M, Gaidano G, Goodman SN, *et al*. Hypermethylation of the DNA repair gene O(6)-methylguanine DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma. J Natl Cancer Inst 2002; 94: 26-32.
8. Katzenellenbogen RA, Baylin SB, Herman JG. Hypermethylation of the DAP-kinase CpG island is a common alteration in B-cell malignancies. Blood 1999; 93: 4347-53.
9. Rossi D, Capello D, Gloghini A, *et al*. Aberrant promoter methylation of multiple genes throughout the clinico-pathologic spectrum of B-cell neoplasia. Haematologica 2004; 89: 154-64.
10. Akhtar M, Cheng Y, Magno RM, *et al*. Promoter methylation regulates Helicobacter pylori-stimulated cyclooxygenase-2 expression in gastric epithelial cells. Cancer Res 2001; 61: 2399-403.
11. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 1996; 93: 9821-6.
12. Kang GH, Lee S, Lee HJ, Hwang KS. Aberrant CpG island hypermethylation of multiple genes in prostate cancer and prostatic intraepithelial neoplasia. J Pathol 2004; 202: 233-40.
13. Ueki T, Toyota M, Sohn T, *et al*. Hypermethylation of multiple genes in pancreatic adenocarcinoma. Cancer Res 2000; 60: 1835-9.
14. Morris MR, Hesson LB, Wagner KJ, *et al*. Multigene methylation analysis of Wilms' tumour and adult renal cell carcinoma. Oncogene 2003; 22: 6794-801.
15. Jaffe ES. Pathology and genetics of tumours of haematopoietic and lymphoid tissues. Lyon: IARC Press; Oxford: Oxford University Press [distributor], 2001; 351 p.
16. Maruyama R, Toyooka S, Toyooka KO, *et al*. Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. Cancer Res 2001; 61: 8659-63.
17. Takahashi T, Shivapurkar N, Reddy J, *et al*. DNA methylation profiles of lymphoid and hematopoietic malignancies. Clin Cancer Res 2004; 10: 2928-35.
18. Li Q, Ahuja N, Burger PC, Issa JP. Methylation and silencing of the Thrombospondin-1 promoter in human cancer. Oncogene 1999; 18: 3284-9.
19. Henrique R, Jeronimo C, Hoque MO, *et al*. MT1G hypermethylation is associated with higher tumor stage in prostate cancer. Cancer Epidemiol Biomarkers Prev 2005; 14: 1274-8.
20. Huang Y, de la Chapelle A, Pellegata NS. Hypermethylation, but not LOH, is associated with the low expression of MT1G and CRABP1 in papillary thyroid carcinoma. Int J Cancer 2003; 104: 735-44.
21. Roth MJ, Abnet CC, Hu N, *et al*. p16, MGMT, RARbeta2, CLDN3, CRBP and MT1G gene methylation in esophageal squamous cell carcinoma and its precursor lesions. Oncol Rep 2006; 15: 1591-7.
22. Li QL, Ito K, Sakakura C, *et al*. Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell 2002; 109: 113-24.
23. Toyota M, Shen L, Ohe-Toyota M, Hamilton SR, Sinicrope FA, Issa JP. Aberrant methylation of the Cyclooxygenase 2 CpG island in colorectal tumors. Cancer Res 2000; 60: 4044-8.
24. Santini V, Kantarjian HM, Issa JP. Changes in DNA methylation in neoplasia: pathophysiology and therapeutic implications. Ann Intern Med 2001; 134: 573-86.